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14. ABSTRACT
Motor recovery following repair of major peripheral nerve injuries is often suboptimal and does not restore adequate functionality. The explanation for these poor results is multi-factorial but certainly "denervation atrophy" plays a significant role. Anabolic steroids, which have been shown to cause hypertrophy of muscle fibers, increase net protein synthesis, a increase satellite cell activity, may offer a novel therapeutic approach to improving muscle recovery following reinnervation of chronically denervated muscle. This hypothesis was tested in a rat model consisting of 8 groups (n=15) made up of three or six month denervated hind limb groups, steroid treatment groups, and sham groups. Evaluation included nerve conduction testing, force contraction measurements, comparative morphology, histolog protein electrophoresis, and immunohistochemical evaluation. Results revealed trends towards increased mass an force production in the steroid treated group (with three months denervation time) but no statistical difference in tl three or six month denervation with steroid treatment groups. Fiber types shifted towards increased amounts of II fiber types with denervation (with this effect augmented in the three month group with steroid treatment) though there was no shift from type I to II fibers. There was no effect of either steroid or denervation on the ratio of satellite cells to muscle fibers. In conclusion, there did not seem to be a functional benefit for anabolic steroid treatment following reversal of prolonged denervation atrophy.

15. SUBJECT TERMS
anabolic steroids, denervation atrophy, muscle reinnervation

Table of Contents

Report: Anabolic Steroid Reversal of Denervation Atrophy

INTRODUCTION:

Functional recovery following major peripheral nerve injuries is often suboptimal despite appropriate and standard treatment. Although a complex and multifactorial problem, time related permanent changes to denervated muscle awaiting reinnervation play a significant role in these poor results. This process, referred to as denervation atrophy, involves loss of muscle mass, muscle fiber size, and contractile composition. While other researchers continue to search for ways to improve nerve regeneration and to avoid or decrease the development of these muscle changes, our focus has been on strategies to optimize and augment the function of reinnervated muscles. The purpose of this study was to investigate the role of anabolic steroids in improving muscle mass and functional recovery strength in reinnervated muscles following the establishment of denervation atrophy. Detailed electrophysiology, immunohistochemistry, and protein electrophoresis was implemented to confirm and add physiological insights to our results.

BODY:

Surgical Manipulation:

Eight groups of immature female Sprague-Dawley rats (n=15; 120 rats total) were used in the study as outlined in Figure 1. Half of the rats underwent 3 months (Groups 1 and 2) or 6 months (Groups 5 and 6) of hind limb denervation (by tibial nerve transection), followed by two months of reinnervation (tibial nerve repaired). After the first month of reinnervation, Groups 1 and 5 were administered supra-physiological doses of anabolic steroids. Assessment of reinnervation and muscle recovery was performed at the end of the recovery period.

The direct effects of anabolic steroids on re-innervated muscle were assessed by comparing Groups 1 and 2, and Groups 5 and 6. Specificity of anabolic steroid affects to reinnervated muscle were assessed by comparing the relative effects of Groups 1 and 2 to Groups 3 and 4, and the relative effects of Groups 5 and 6 to Groups 7 and 8.

Animals in the Experimental (steroid) (Groups 1 and 5) and Control groups (2 and 6) underwent identical surgical manipulations. Under general isofluorane anesthesia the left sciatic nerve was exposed via a standard biceps femoris semi-tendinosis muscle splitting approach and the tibial nerve transected just past the bifurcation. The two nerve ends were separated and buried in muscle bellies to prevent inadvertent nerve regeneration. Sham groups (3,4,7,and 8) underwent sham operations in which nerves were exposed but not transected. At either 3 months (Groups 1-4) or 6 months (Groups 5-8), the rats underwent a second survival surgery. The left hind limb was reopened and the sciatic nerve and its divisions re-exposed. The transected tibial nerves were dissected out and repaired using 1 cm of tibial nerve autograft harvested from the contra lateral leg

(to avoid tension) using standard microsurgical techniques (two or three epineural 10-0 nylon sutures per repair site). After 1 month of nerve regeneration time (to allow axons to regenerate to the muscles), all groups underwent placement of a subcutaneous osmotic pump and were administered a continuous infusion of either anabolic steroid in sesame oil (Groups 1,3,5,and 7) or sesame oil (Groups 2, 4, 6 and 8) for one month.

In vivo testing was performed at the end of the regeneration period (after one month of "treatment" with steroid or sesame oil carrier only).

Muscle Force

Following anesthesia, animal limbs were affixed to a Teflon operating board using umbilical tape tied to posts screwed into the board. The sciatic nerve was exposed and platinum electrodes inserted 1 cm proximal to the graft position. The platinum electrodes were connected to a Grass stimulator model SD9 (Astro-Med Inc., West Warwick, RI). Stimulation was performed using a 2 ms duration and 2 ms delay at varying voltages and frequencies.

To measure only gastrocnemius force, the knee joint was immobilized by placing a nail through the center of the joint into a pre-drilled hole in the operating board. The ankle joint was similarly immobilized. The Achilles tendon was exposed and cut free from the calcaneous and tied with 2-O silk sutures. The free end of the suture was connected to a MLT500/A force transducer (AD Instruments, Inc., Colorado Springs, CO). Output of the force transducer was collected digitally using a MAC computer and AD Instruments and software provided by the manufacturer (AD Instruments, Inc.). The force transducer output is in millivolts. Calibration was accomplished using known

masses and the assumed acceleration of gravity of 9.81 m s^2. The output was almost exactly 1 mV = 1 N.

Twitch parameters were sought under conditions of maximum recruitment and optimum length. The sciatic nerve was first stimulated at single pulses at a frequency of 0.5 Hz for stimulation voltages varying between 1-5 V. The minimum voltage giving plateau twitch force was used for subsequent studies.

The tetanic frequency was found by increasing the frequency of maximum stimulation in 5 Hz increments from 10 to 60 Hz. The length at maximum tetanic force was found by increasing the length of the muscle in 0.5 mm increments until active force began to decline.

At this time the muscle was shortened to the maximum length and a series of twitches were made. The maximum twitch force, time to reach one-half of maximum force, and time to decay from maximum to one-half of twitch force were obtained from the force record off-line.

Electrophysiology

Direct nerve-to-nerve and nerve to muscle nerve conduction studies were performed. A Nicolet Viking EMG machine was used to obtain the responses. A non-recurrent stimulation at a duration of 0.5 ms was used. The intensity of the stimulation was slowly increased until a maximal amplitude was achieved. This stimulation was performed a second time to ensure reproducibility. The baseline to peak amplitude was measured for each response. Onset and peak latencies were measured for each nerve-to-nerve stimulation.

Immunohistochemistry

The gastrocnemius muscle was removed, weighed and immersed in TBS Tissue Freezing Medium (Medical Sciences, Inc. , Durham, NC), maintaining medial and lateral orientation and in vivo length in a plastic histology mold (TedPella, Inc. Redding, CA). The embedded muscle was rapidly frozen by plunging into isopentane cooled in liquid nitrogen for about 30 seconds and stored in a freezer at -70° F. A five mm block was cut transversely from each muscle about five mm distal to its origin. Serial 10 μm transverse cryostat sections were cut from each muscle block, collected on Fisher Superfrost Plus microscope slides (Fisher Scientific, Suwanee, GA) and air dried before being returned to the -70° freezer.

Mouse monoclonal antisera was used for immunostaining, including WB-MHs antibodies raised against rat MHC I (Vector labs, Butlingame, CA), SC-71 antibodies raised against rat MHC 2a (Developmental Studies Hybridoma Bank, Univ. Iowa), and BF-F3 antibodies raised against rat MHC 2b (cell line obtained from ATCC, Manassas, VA). Muscle sections were placed in 10mM phosphate buffered saline (PBS, pH 7.5). Non-specific binding was blocked by a 20-minute incubation period in 10% normal blocking serum. The sections were incubated with diluted (1:50) primary antibody for 1 hour, rinsed with PBS and incubated with diluted (1:1000) biotinylated secondary antibody for 30 minutes. After rinsing in PBS, the sections were reacted with Vectastain® Elite ABC Reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, followed by another wash in 10mM PBS. A diaminobenzadine solution was used for visualization (Vector DAB kit, Vector Laboratories). The stained sections were dehydrated in ascending alcohols, cleared in xylene and mounted in permount. An

Image-Pro®Plus image analysis system with a Nikon Microphot-7xA Microscope and a Q Imaging digital camera (Media Cybernetics®, Silver Spring, MD), appropriately calibrated for 10X objective magnification was used to analyze the minimum diameter and area of muscle fibers, using Image-Pro v. 7.01 image analysis software. Four sections were chosen at random from each muscle section providing a minimum of 100 positive fibers from each section.

Stem Cell Immunostaining and Analysis:

Fresh frozen cryo-sections were allowed to air-dry for 15 minutes before tissue was fixed by incubating in 4% paraformaldehyde (in PBS) for 15 minutes. Sections were washed several times in PBS to remove any residual fixative and then was incubated with blocking buffer (2.5% normal goat serum, 5% bovine serum albumin, 0.2% Triton-X100 in PBS) for 30 minutes. Primary antibodies (anti-entactin [Chemicon]; anti-M-cadherin [Abcam, Inc.]) diluted in blocking buffer, were incubated on the sections for 12 hours at 4°C, then sections were washed several times with PBS. Secondary antibodies (Invitrogen/Millipore), diluted in blocking buffer, were then incubated on the slides for 1 hour at room temperature. Sections were washed thoroughly with PBS, incubated in DAPI solution, cover-slipped with VectaShield, and visualized on an Zeis AxioImager. All analysis was performed blind. At least 10 random images were acquired with identical settings for each immunostained sections. For each image the number of muscles fibers (labeled by entactin-immunoreactivity in the basal lamina that surrounds each muscle fiber) and satellite cells (identified by M-cadherin immunoreactivity) were counted manually. Sample identification remained in code (preparation by blinded observer) until all samples were analyzed and all data collected.

Electrophoresis of Myosin Heavy Chains

The remainder of the medial gastrocnemius muscle was used for differential MHC analysis. Frozen muscles were lyophilized, minced with scissors and homogenized with a pellet pestle in ice-cold extraction buffer (0.3 M NaCl, 0.15 M Na_2HPO_4, 10 mM EDTA, pH 6.5). The solution was agitated and stirred at 4ºC for 60 minutes and centrifuged (10,000 X gravity) for 10 minutes. The total protein concentrations of the supernatants were determined using the Bio-Rad protein assay for microtiter plates (Bio Rad Laboratories, Hercules, CA), based on the Bradford dye-binding procedure. The supernatants were diluted to 0.25 mg/ml in extraction buffer and stored at -70ºC. MHC isoforms were separated using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. This technique enables the separation of the developmental MHC isoforms from the three or four adult MHC isoforms typically expressed in rat skeletal muscles.

Gel slabs (0.75 mm thick) consisted of a 13.5 cm 8% separating gel and a 4 cm 4% stacking gel. All gels were made from the same stock solutions and all chemicals were of electrophoresis grade. A 2X Laemmli sample buffer (22) was added to the muscle samples to yield a final protein concentration of 0.125 mg/ml. Samples were boiled for 5 minutes to denature the protein. Each lane on a gel was loaded with 20 µl of a muscle sample. Tris-glycine-SDS running buffers cooled to 4ºC were used. Electrophoresis was performed using a vertical slab gel unit (Protean II xi Cell, Bio Rad Laboratories) run at 275 V for 30 hours at 4ºC.

Separating gels were silver stained using the Silver Stain Plus Kit (Bio Rad Laboratories). Images of silver-stained gels were obtained using an AGFA Duoscan HiD scanner (AGFA Corporation, Ridgefield Park, NJ). Relative amounts of MHC isoforms were determined using Gel-Pro® Analyzer (Media Cybernetics®, Silver Spring, MD), image analysis software.

Statistical Analysis

Statistical analysis of immunochemistry of muscle fibers was carried out using IBM SPSS v. 19 software package. A one-way analysis of variance (ANOVA) was used to analyze the results of the muscle masses, fiber type distributions, and diameters.

Statistical analysis of muscle contraction, nerve electrophysiology, and muscle morphology was performed using GraphPad Prism Software (La Jolla, CA) and calculated using a one-way analysis of variance (ANOVA) among all groups with Tukey's post comparison test.

Statistical analysis for stem cell counts was performed using IBM IBM SPSS v. 19 software package and calculated using Tukey-Kramer Test for Differences Between means and Student's t-test to compare ratios between individual groups.

RESULTS

Muscle Force:

Muscle mass was significantly depressed in all nerve-transections compared to their sham-operated controls. Steroid administration had no significant effect on muscle mass in either the nerve-transected or in the sham-operated controls. There were no significant differences in muscle mass between the groups at three months or six months post-transection (Figure 2). The maximum twitch force in muscles from transected animals was reduced in proportion to the muscle mass. Steroid administration tended to increase the maximum twitch force for the three month denervation groups, but this effect did not reach statistical significance ($p= 0.16$) (Figure 3). Specific force (force per muscle mass) showed no significant differences between experimental groups or between sham groups (Figure 4).

Half time to peak tension is defined as the time from the start of the tension transient to the time at which tension reached one-half of the maximum twitch tension. At three months post-transection this varied from 13 to 15 ms and there were no significant differences among the transection and sham groups or in the steroid verses control groups (Figure 5). Similarly, half-time of force relaxation showed no differences between any of the treatments (Figure 6). Denervation and subsequent grafting were no different from sham-operated controls. Steroids administration had no effect on either half-time of force development or half-time of relaxation.

Electrophysiology:

Nerve action potentials were recorded from the sciatic nerve. A prolonged latency was recorded for both Groups 1 and 5, when compared with Group 7 ($p < 0.5$). A similar prolonged latency was recorded from both Groups 2 and 6, when compared to

group 7 ($p < 0.5$). There was no statistical difference in latency when steroids (group 1 and 5) were applied to the transected nerve when compared to PG (group 2 and 6) suggesting that steroids had no affect on the speed of remyelination of the nerve (Figure 7). Similar trends are seen with amplitude, as again, results were recordable. A significantly lower amplitude was recorded from group 4 when compared to groups 2, 5, and 6 ($p < 0.5$) suggesting that although reinnervation has commenced, t has not reached the amplitude of a nerve which had not been transected (Figure 8).

Histology:

Diameters of muscle fibers by type:

Table 1 lists the diameters and cross sectional areas of IIa, IIx, IIb, and type I muscle fibers. Considering that the type IIb should be the strongest and fastest of the muscle fiber types, it follows that they should have the largest muscle fibers. Comparing groups 1 and 2, the size of group 1 type IIb is significantly larger in diameter ($p<.001$) and area ($p<.002$) than group 2. But when compared to normal, the size of group 1 MHC type IIb remains significantly smaller than normal (group 4) (diameter ($p< .001$) and area ($p < .05$)). This is also true for the other muscle fiber types when compared to normal but not at a significant level.

The diameters of muscle fiber types IIa ($p< .05$) and IIb ($p< .001$) of the group 5 remain significantly smaller than group 8 (normal). The steroids did not have a significant effect on the diameters of the group 5 compared to group 6 though type IIx and IIb with steroids tended to be larger than those without steroids. Slow type muscle

fibers (type I) were slightly smaller in group 5 relative to group 6, consistent with the difference between groups 7 and 8 but the difference is not significant.

Proportions of muscle fiber types shown by electrophoresis:

As demonstrated in Table 2, groups 1 and 2 have significantly smaller proportion of muscle fiber type IIb and IIx and significantly more IIa than normal (group 4, $p < .001$). The muscles with peripheral nerve injury (group 1) showed a further significant reduction of type IIb ($p < .001$) and more type IIa ($p < .001$), when the steroid was added. Note that there is expression of the developmental isoform of the MHC during this initial time frame which may draw away from the type IIa and type IIb isoforms.

Groups 5 and 6 still have significantly less muscle fiber type IIb ($p < .001$) and significantly more slow type muscle fibers ($p < .001$) than normal (group 8). The proportion of IIx and IIa are not significantly different from normal.

Stem cell analysis:

Table 3 shows the ratio of satellite cells to muscle fibers for each experimental condition. The proportion of satellite cells to muscle fibers was not statistically changed at each age of treatment regardless of whether muscles had been denervated, received steroid treatment, or both (compare data for groups 1-4 or for groups 5-8). A Tukey-Kramer Test for Differences Between Means revealed there were no significant differences between the mean ratio of satellite cells to muscle fibers from groups 1-4 (p value for pair comparisons of these group were all greater than $p=0.71$). Likewise differences between the mean ratio of satellite cells

to muscle fibers for groups 5-8 were not significant (p value for pair comparisons of these group were all greater than p=0.87). A significant difference was observed when comparing the mean ratio of satellite cells to muscle fibers from steroid treated muscles that were denervated for 3 months (group 1), versus those denervated for 6 months (group 5)(group 1 vs group 5 – p=0.0083; group 1 vs. 6 – p=0.16; group 1 vs. group 7 – p=0.04; group 1 vs. group 8 – p=0.08 by Tukey-Kramer Test for Differences Between means). Comparison of all other mean ratios between individual groups was not significant. However an overall age dependent increase in the ratio of satellite cells to muscle fibers was observed (average ratio of groups 1-4 = 1.43 +/- 0.09 [SD] vs. average ratio of groups 5-8 = 1.70+/- 0.07[SD]; p=0.002 by Students t-test).

Discussion of results

Motor recovery following repair of major peripheral nerve injuries as often seen in both civilian and military trauma is often suboptimal and does not restore adequate functionality[1-3]. The explanation for these poor results is multi-factorial but certainly "denervation atrophy" plays a significant role[4]. This effect is exacerbated by delays (either due to late surgical intervention or to prolonged regeneration lengths) in re-establishing motor axonal connections to the muscle fibers themselves. The correlation of loss of muscle mass, muscle fiber size, and contractile composition of chronically denervated muscle, even when reinnervated, has been well established for over half a century. The pathophysiology of this phenomenon is not fully understood but includes diminished protein synthesis and loss of satellite cells. Satellite cells have a prominent

role in muscle fiber regeneration. These normally quiescent cells are up-regulated to divide and proliferate [5-7] before being incorporated into damaged or denervated muscle fibers[8, 9]. This ability to self-replicate is limited, however, and the eventual exhaustion of this population is linked to the development of "irreversible denervation atrophy" [4, 5, 10].

Most strategies for improving clinical outcomes have focused on limiting denervation atrophy primarily by expediting and improving axonal regeneration. Although these concepts are important, the idea of finding a treatment to reverse the atrophy once it had occurred offered a new and innovative approach to an unsolved problem. Anabolic steroids seemed to offer the most obvious choice for an initial investigation. Anabolic steroids, which are basically synthetic forms of testosterone designed to maximize anabolic effects while minimizing androgenic side-effects, may have a potential role in reversing denervation atrophy. Salmons demonstrated improved size and strength in the tibialis anterior muscle of female rabbits following 12 weeks anabolic steroid administration [11]. Taylor et al. found a definite anabolic response in a sedentary rabbit model treated with nandrolone decanoate[12] and Zhao et al. found that nandrolone significantly reduced chronic denervation atrophy (but not acute atrophy) in rats when administered 28 days post nerve transection[13]. In certain types of atrophy, including disuse, HIV, and sarcopenia, anabolic steroids have been shown to have a positive effect[14, 15]. When given at supra-physiological doses, anabolic steroids, increase protein synthesis, decrease protein breakdown, and their effects have been attributed to higher ribosomal content in muscle cells [16-18]. The observed concurrent increase in the number of nuclei in these enlarged muscle cells as well as a concurrent increase in the satellite cell population suggests that at least one mechanism of anabolic steroid induced

hypertrophy is increased integration of satellite cells in addition to the increased protein synthesis. There is some evidence that pluripotent stem cells are stimulated to increase satellite cell populations, as well[19].

Despite what appears to be promising literature support for choosing anabolic steroids to test our concept of augmenting reinnervated muscle cells, our results are remarkably consistent in failing to demonstrate any significant effect from this treatment. Although a positive trend was noted, neither reinnervated muscle nor normal (sham muscle) showed a statistically significant increase in peak muscle force following steroid treatment. Attempts to "tease out" more subtle effects were also disappointing, as time to force development, time to relaxation, and specific force measurement (force generation based on muscle weight) also failed to demonstrate significant positive effects. As expected by these findings, indirect measures including muscle mass (weight and diameter), muscle cell size, and muscle fiber type all failed to support significant anabolic effect.

In trying to analyze these surprising results, the most important question is whether the concept of augmenting reinnervated muscle is flawed or whether nandrolone (at the dosage given) was an ineffective treatment choice. Our failure to see a significant effect from anabolic steroid treatment even in our sham groups might point towards the "ineffective treatment choice" explanation. While it is widely accepted that administration of testosterone (or anabolic steroids) in situations of low levels of circulating testosterone (i.e. associated with castration) will result in muscle hypertrophy (or at least correction of atrophy), there is controversy regarding similar effects in subjects with normal levels of testosterone (or lacking a deficit such as females)[20, 21]. In

contrast to studies already described above, Bartsch et al. and Bates et al. both reported no increased muscle growth following administration of supraphysiological doses of anabolic or natural steroids to normal rats[22, 23]. Egginton administered nandrolone to sedentary female rats for six weeks and found that while there was a generalized 20% increase in weight, muscle force gains could not be demonstrated[24]. Possible explanations for these seemingly contrasting results include varying effects across species, muscle groups, and study design [21, 25, 26].

Furthermore, there is no consensus regarding treatment dose or duration of treatment. The nandrolone dosing in our study of 6mg/kg/week was much higher than the Food and Drug Administration recommended analogous dosing for a human[27], but comparable to other animal studies (5.6mg/kg/day[24], 6mg/kg/week[28-30], 3.75mg/kg/week[31]). Some studies, however, used even higher dosing (15mg/kg/week[12, 32]), and body builders are reported to use 10 to 100 times the normal therapeutic dosing of anabolic steroids[33]. Another possible explanation of our study results may, therefore, be as simple as inadequate dosing. Likewise, our treatment period of 4 weeks was within the range of other studies (2 to 8 weeks[12], 5 to 6 weeks[24], 30 days[34], 28 days[13], 14 days[35] , 2.5 weeks[28], 4 weeks[29]) though still on the conservative side. Salmons, in particular, reported positive effects of anabolic steroids at 12 weeks but not at 4 weeks[11].

The lack of efficacy of steroid treatment in normal muscles, however, does not mandate a lack of efficacy of the same treatment following reinnervation of the moderately or severely atrophic muscles. While there are studies demonstrating steroid related delays in onset and delayed progression of atrophy associated with denervation[13] or disuse atrophy[32] (both catabolic states), our concept was to augment the

physiologically anabolic state (and reversal of a catabolic state) associated with reinnervation. In other words, it is possible that anabolic steroid treatment could have a more obvious effect when given during analogous states of muscle healing or growth. Indeed, quicker and more robust healing of contused or injured muscles has been associated with steroid treatment[30, 35]. Along this same rationale, anabolic steroids may only potentiate muscle growth and strengthening. Elashoff et al. in a meta-analysis of human anabolic steroid use studies concluded that there was no evidence to support a strength enhancing effect in the non-strength-training subjects[33].

Since we expected to see a positive effect from the steroid treatment, our study included more detailed exploration of denervation and anabolic steroid induced structural and physiologic muscle changes including shifts in muscle fiber size, muscle fiber type, and stem cell population. Muscle fibers are broadly categorized as type I or II based on the muscle fibers' metabolism (type I is aerobic and II is anaerobic). Type I fibers have greater endurance but are weaker and Type II fibers are capable of generating more force but fatigue quickly. Type II fibers are subtyped based on myosin isoforms as IIa, IIx, and IIb which also reflect (in this order) increasing force producing capability (IIb is highest), increasing contraction speed (IIb is fastest), and decreasing resistance to fatigue (IIb fatigues quickest). In other words, muscle with an increased size and percentage of IIb fibers would theoretically represent a muscle capable of producing greater force but for only short periods of time. As expected in our study, all temporarily denervated groups (both treated and sham) demonstrated smaller average muscle fiber diameters than non-denervated muscles. Force is proportional to cross-sectional area, so weaker muscles would be expected to have smaller fibers.

Based just on size of the fibers, there appeared to be some reversal of atrophy with or without anabolic steroid treatment. In the three month sham verses denervation groups the average muscle fiber size was only 2.2 times larger. The same comparison for the six months groups was 1.7 times larger (which would suggest at least some degree of increased atrophy). In small mammal experiments in which chronic denervation is not reversed, fiber sizes are decreased 80% (for equivalent time periods)[36].

Previous studies have demonstrated that when muscle is denervated, initially there seems to be a disproportionate effect on larger muscle fibers such as the IIb fibers-- these atrophy first[37]. With prolonged denervation, however, muscle fibers are throught to shift from predominantly type I to predominantly type II---and especially immature isoforms of type II fibers[38, 39]. This slow to fast fiber transition is typically maintained following reinnervation[40]. We did not see a significant shift from type I to II fibers but among type II fibers there was a see a shift towards type IIa for both the three month and six month denervation groups.

Testosterone and anabolic steroids have previously been shown to induce muscle fiber hypertrophy in both type I and II fibers[15, 41]. The exact mechanism is not entirely worked out but theories include a direct effect on net protein synthesis[16, 18], an increase is stem cell activity[15], or both. Fiber type proportions are not thought to change[15]. Although we saw an increase in IIb fiber diameter in the steroid treated 3 month reinnervation group (which would technically be considered hypertrophy), the proportion of IIb myosin heavy chain proteins actually decreased, and there was a further increase proportion of IIa proteins associated with steroid treatment. For the six month reinnervated rats, this proportional drop in IIb fibers was again noted but this time there

was no difference between steroid treated and untreated rats. These results are hard to interpret and for both reinnervated and sham treated rats are in contrast to previous reports (though these focused more on size that protein levels). The overall effect was an increase in treated muscle IIb diameters with a net increase in IIa proteins (not IIb) which implies larger but fewer IIb fibers and more IIa fibers.

In light of our failure to demonstrate significant muscle size or strength changes associated with anabolic steroid administration, the lack of a proportional increase in satellite cells to muscle fibers in our experimental groups is not surprising. The lack of any effect of the anabolic steroid on satellite cell activity even in the sham groups, however, is in direct contrast to studies by Joubert and Tobin[42] and Sinha-Hikim et al[43]., though there are some important differences between their work and our study. Joubert and Tobin demonstrated testosterone induced increased satellite cell activity in the rat levator ani muscle which is much more androgen sensitive than the muscles tested in our study[42]. Similarly, Sinha-Hikim's group evaluated aged human muscle. Anabolic steroids may not illicit the same response in the rat gastrocnemius muscle[20]. There is a similar problem of comparison with the existing literature regarding the untreated "re-innervation group". While in acutely denervated muscle, satellite cell activity increases[44] and in chronically denervated muscle, satellite cell activity decreases[45], it is not known what happens to the net satellite cell activity after two months of re-innervation. Our results suggest that this cell activity returns to the baseline of normal age matched controls. Had we tested at multiple time points, we may have noted a net decrease in satellite cell activity as the denervated state of the muscle had gradually reversed. Similarly, the reversal of the denervated atrophic state (and the associated level

of satellite cell activity) may be already maxed out with the re-innervation process and any potential augmenting effects of the anabolic steroids may have been inconsequential. Nnodim was unable to demonstrate any additional effect of castration on satellite cell counts when compared to denervation alone[19]. Finally, the age dependent increase in satellite cell activity across all groups may be a reflection of our labeling technique. M-cadherin (the target of our immunostaining) is an established marker for rodent satellite cells[46]. While, a general decrease in satellite cell activity would be expected with aging of the rodents, the diverse and heterogenous population of satellite cells may not be fully captured with this single label[47].

Our findings, in fact, are so out of proportion from what we expected, that we need to question the validity of our animal model. The goal of our model was to create either moderate or severe atrophy, reverse it by restoring innervation, and then test if anabolic steroid treatment would improve muscle recovery. The initiation of the treatment protocol was delayed one month after nerve repair specifically to avoid any potential steroid influences on nerve regeneration (increased nerve regeneration following anabolic steroid has been previously demonstrated[48-50]). Our nerve conduction data suggests that this was not a factor. However, after three or six months of denervation, we should have seen much weaker and atrophic muscles than what we found. Kobayashi et al. found a "precipitous and profound decrease in the recovery of both muscle mass and integrated motor function if the [nerve] reconstruction was delayed for longer than 1 month" in a rodent sciatic nerve repair model[10]. In a similar model, Aydin et al.

found nerve repairs delayed for a month or longer resulted in a 30% to 50% force deficit[51].

We cannot explain these discrepancies with the scientific literature but will note that our results, while unexpected and hard to explain, are remarkably consistent across a spectrum of different assessment tools and investigators. Atrophic changes were present but did not effect force generation, muscle mass, fiber size, or muscle fiber constitution as profoundly as expected. Anabolic steroids had minimal or no effects on force contraction, mass, fiber size, or muscle fiber constitution.

KEY RESEARCH ACCOMPLISHMENTS:

- At acceptable dosages, anabolic steroids do not seem to have a potential role in augmenting muscle recovery following delayed reinnervation

REPORTABLE OUTCOMES:

- Manuscript pending completion of satellite cell counts
- Will plan to submit to national peripheral nerve meeting (American Society for Peripheral Nerve)
- Poster presentation at the annual meeting of the Society for Neuroscience.
    - *M. S. SHALL[1], J. FEHER[2], M. FOX[3], S. MALLU[4], J. E. ISAACS: Anabolic steroid effects on denervation atrophy in rats. Annual Meeting of the Society for Neuroscience, 2011

CONCLUSION:

In this rodent model, nandrolone when administered through a continuous transfusion osmotic pump at a fixed rate of 6mg/kg/week for four weeks did not have a significant effect on muscle recovery following reinnervation after either three or six months prolonged denervation. Although there may be a role of mega-dose steroids for this application, the potential dangers associated with this class of drugs at those doses would realistically prevent translation to the clinical setting. The idea of augmenting weakened reinnervated muscles with a more potent anabolic agent is still viable and would offer enormous clinical benefit to both military and non-military victims of trauma to the peripheral nervous system. Potential agents to be considered for future research include follistatin, myostatin blocking agents, growth hormones, or a combination (including anabolic steroids).

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APPENDIX

FIGURES

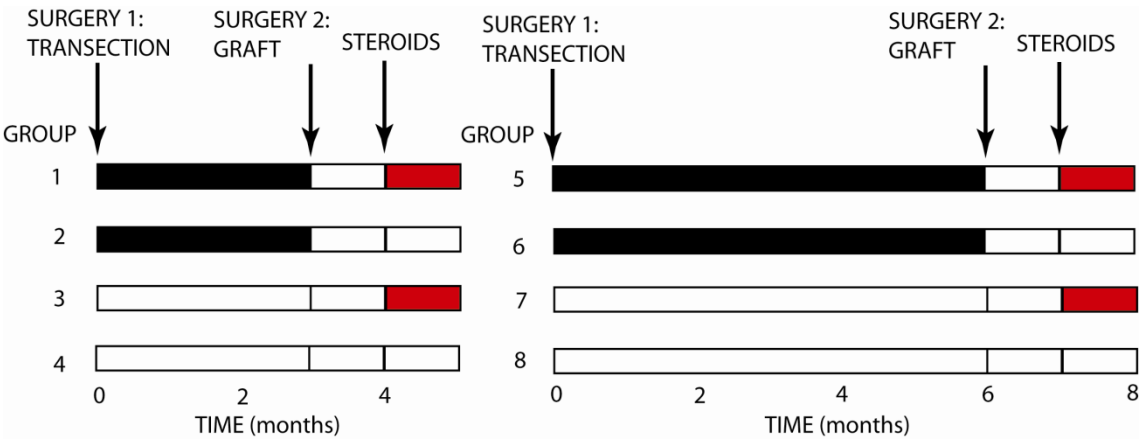

Fig. 1. Black indicates period of denervation. White indicates period of sham operation or period of reinnervation. Red indicates period of steroid administration (6 mg/kg/week of nandrolone delivered by Alzet osmotic infusion pumps (Durect Corporation, Cupertino, CA) implanted subcutaneously).

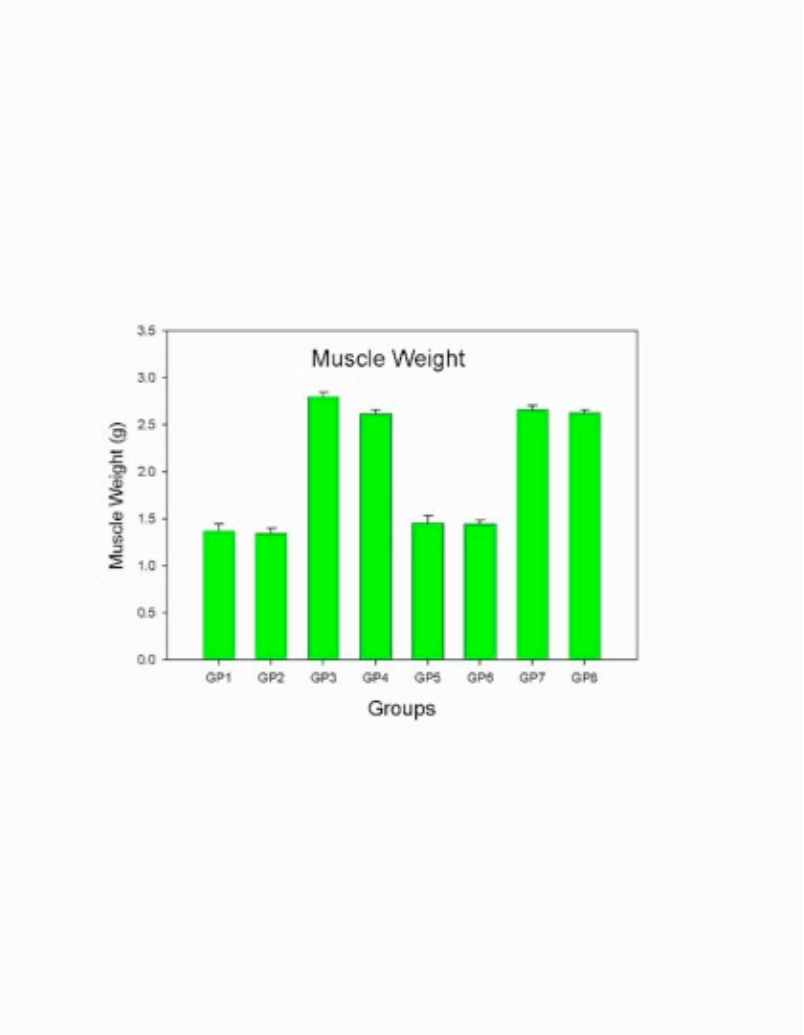

Fig. 2. Muscle weight for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

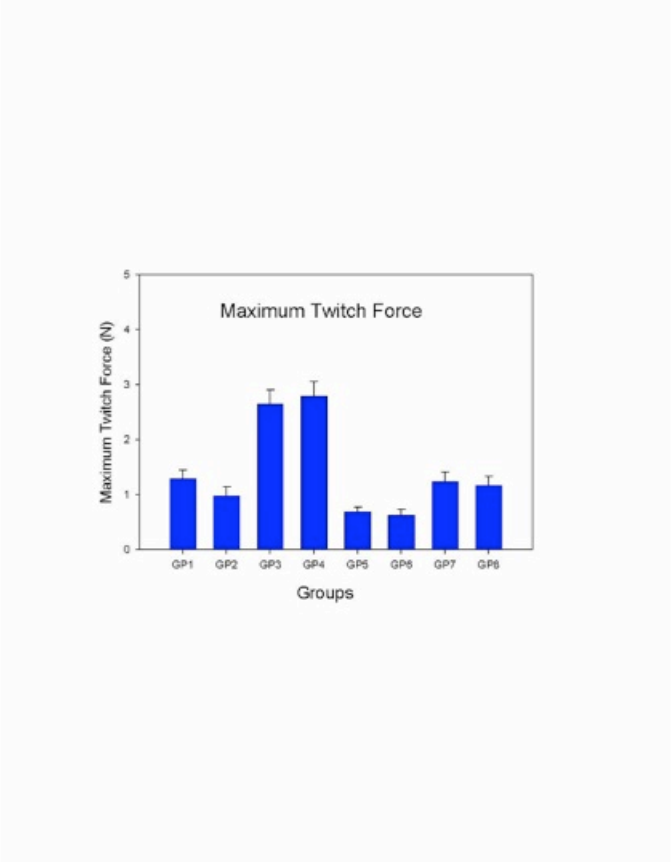

Figure 3. Maximum twitch forces for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

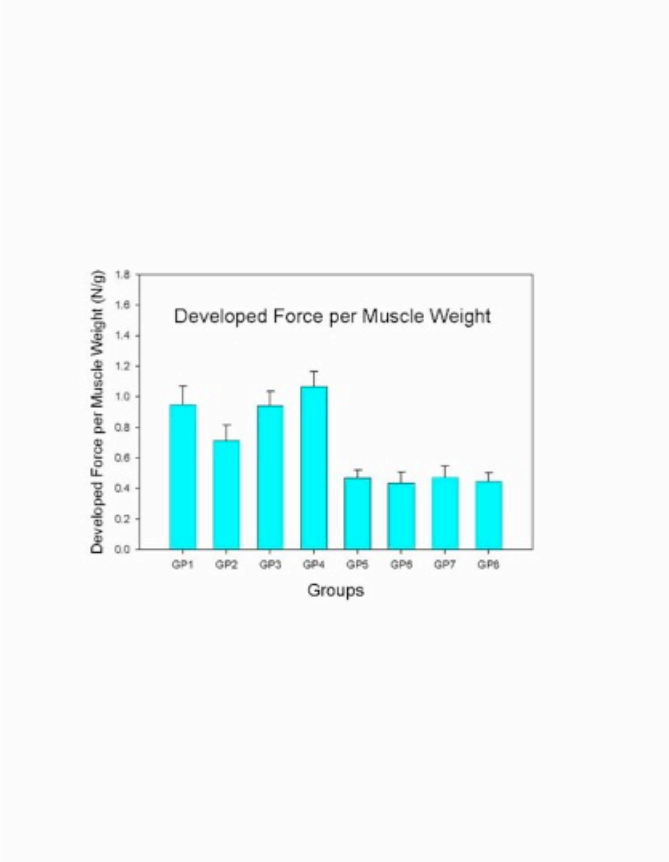

Figure 4. Specific Force for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

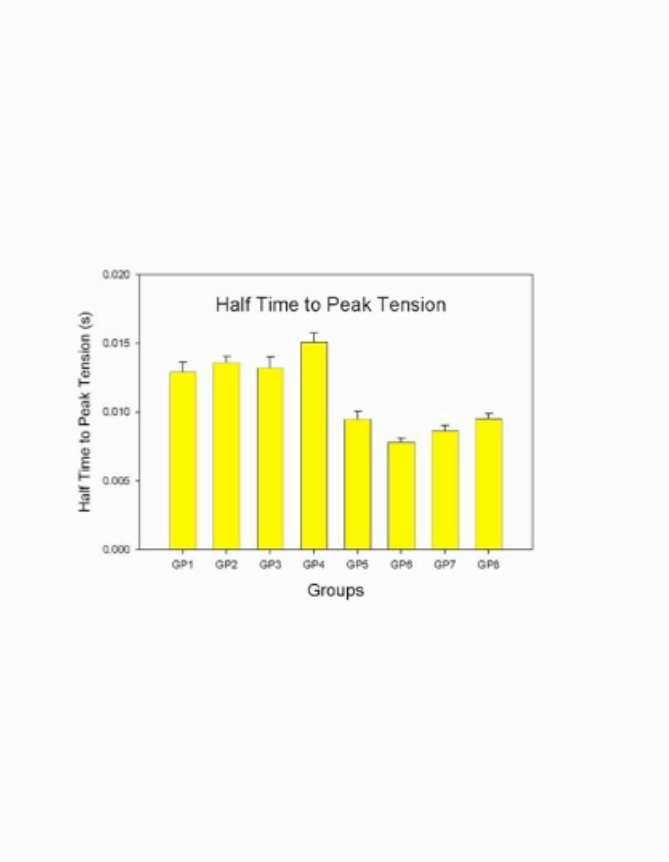

Figure 5. Half time to peak tension for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

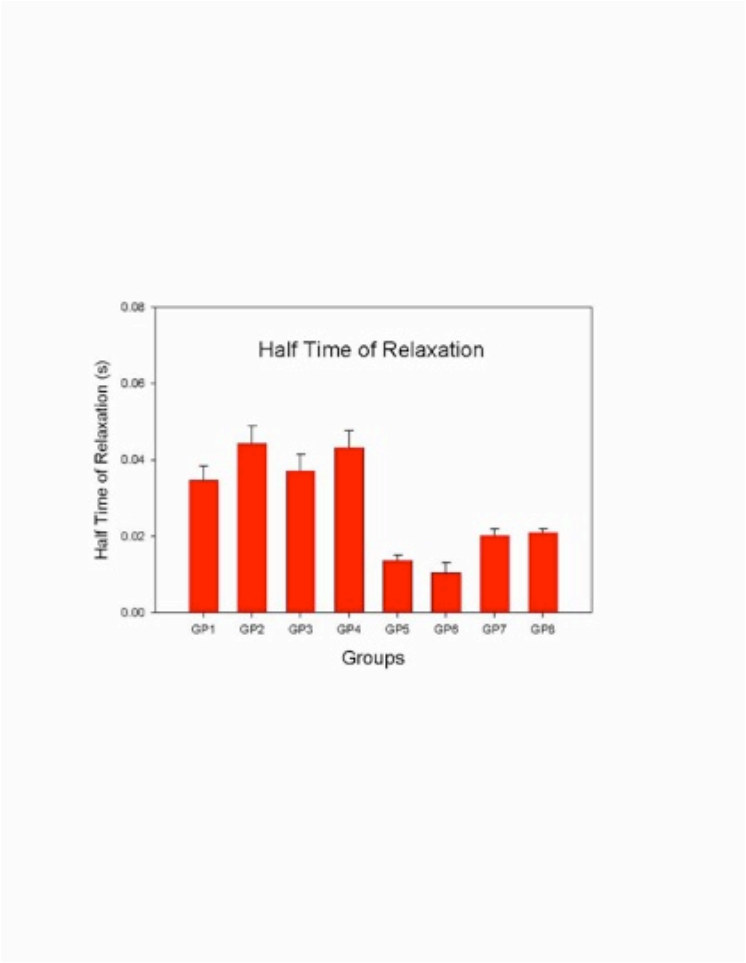

Figure 6. Half time of relaxation for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

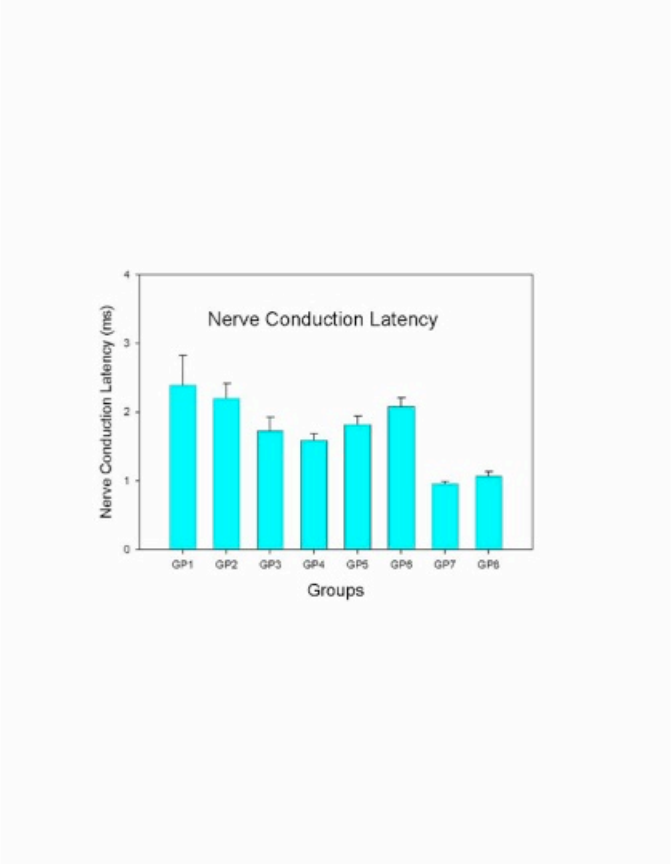

Figure 7. Nerve conduction latency for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

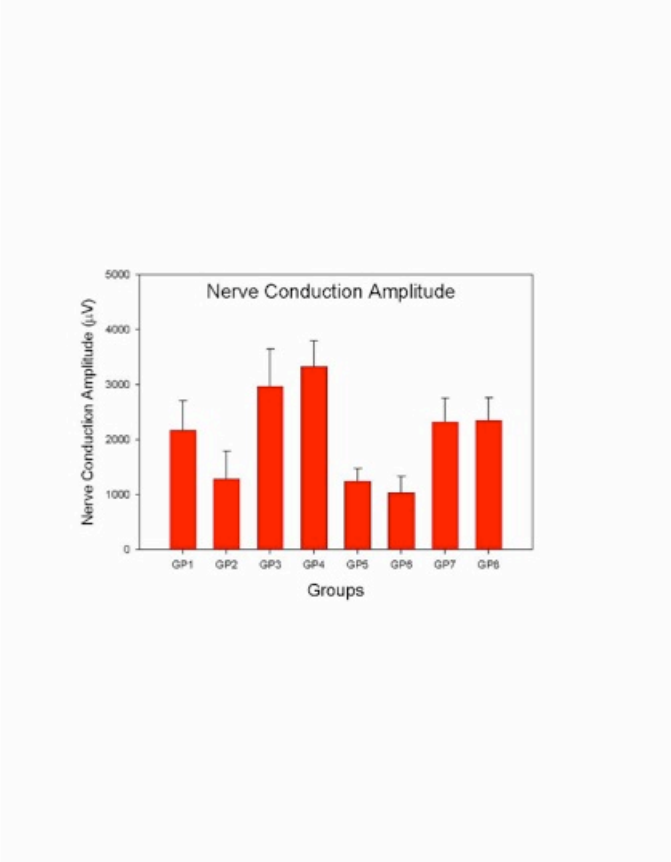

Figure 8. Nerve conduction amplitudes for the 8 groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

TABLES

	Diameters				
	IIa	IIx	IIb	I	average
Group 1	29.1±12.4	35.0±10.5	30.4±13.4	34.5±13.8	32.25
Group 2	29.3±10.9	31.8±9.6	27.9±12.8	35.4±10.6	31.1
Group 3	41.9±7.8	44.8±10.7	52.6±12.4	43.4±9.0	45.675
Group 4	40.1±7.8	42.8±11.4	51.7±14.3	41.9±14.7	44.125
Group 5	35.7±9.8	37.5±10.8	38.5±13.2	35.2±12.3	36.725
Group 6	35.7±9.8	36.8±10.7	36.8±13.0	38.4±8.8	36.925
Group 7	58.0±20.8	44.1±11.2	63.3±13.7	45.9±8.6	52.9
Group 8	44.0±8.8	47.9±10.4	59.6±13.5	48.8±9.3	50.075
	areas				
Group 1	1357.5±1047.9	1949.7±1128.7	1500.0±1244.4	1962.7±1110.4	1689.975
Group 2	1311.5±873.0	1457.7±779.3	1341.0±1170.6	1790.7±866.2	1475.225
Group 3	2467.8±661.6	3083.9±1241.4	4245.9±1705.0	2708.0±950.1	3126.4
Group 4	2313.2±768.6	2716.4±1275.5	4121.0±1927.3	3411.5±2870.9	3140.525
Group 5	1999.0±927.6	2143.7±1033.8	2330.7±1401.9	1951.6±1067	2106.25
Group 6	2004.9±930.8	2111.5±1143.3	2222.9±1653.3	2158.9±822.9	2124.55
Group 7	2545.6±807.7	3012.3±1217.6	6483.8±2836.0	2966.2±927.2	3726.975
Group 8	2728.7±839.9	3372.0±1194.3	5327.2±2499.6	3306.5±1055.5	3683.6

Table 1- Diameter and areas of muscle fibers broken down by muscle fiber types. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

	devel	IIa	IIx	IIb	I
group 1	15.51±9.4	48.2±10.8	13.2±8.7	15.4±5.2	7.6±5.7
group 2	9.0±4.4	38.9±6.2	12.3±5.0	28.0±5.5	11.7±3.1
group 3		20.8±7.3	13.1±3.1	53.5±6.8	12.6±6.7
group 4		18.9±8	25.2±6.1	45±9.4	11±3.4
group 5		14.4±4.6	32.6±6.3	34.6±9.1	18.3±5.5
group 6		16.2±6.0	29.0±9. 1	35.6±9.6	19.2±11.2
group 7		14.9±4.5	26.3±11.2	47.7±10.2	11.0±3.8
group 8		12.2±3.2	35.2±3.2	40.7±3.4	11.9±3.4

Table 2 Proportions of muscle fibers broken down by muscle fiber types. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting.

	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**
Average	1.302697845	1.518821201	1.482337159	1.435892252	1.783009142	1.624589036	1.733717054	1.652302316
SD	0.33251079	0.397657113	0.295363154	0.459367624	0.367718012	0.270055908	0.215124825	0.283517602
n *	11	9	21	3	11	13	9	14
SEM	0.100255775	0.132552371	0.064453524	0.265216021	0.110871152	0.074900032	0.071708275	0.075773266

* each n represents data from at least 10 random image fields and all analysis was performed blind

Table 3- Average ratio of satellite cells to muscle fibers broken down by groups. Groups 1,2,5, and 6 were transected; Groups 3,4,7 and 8 were sham-operated. Groups 1, 3, 5 and 7 received steroids (6 mg/kg per wk by osmotic pump); Groups 2,4,6, and 8 received sesame oil vehicle by osmotic pump; Groups 1-4 were denervated for 3 months prior to grafting; Groups 5-8 were denervated for 6 months prior to grafting. SD= Standard Deviation; n= sample size; SEM= Standard Error of Measurement.